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Award Number: DAMD17-00-1-0353

TITLE: Microarray technology to study the role of genetic polymorphisms in breast cancer risk

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REPORT DATE: July 2004

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
               Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
                        Distribution Unlimited

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20041118 059

REPORT DOCUMENTATION PAGE

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE July 2004	3. REPORT TYPE AND DATES COVERED Final (1 Jun 00 – 1 Jun 04)

4. TITLE AND SUBTITLE	5. FUNDING NUMBERS
Microarray technology to study the role of genetic polymorphisms in breast cancer risk	DAMD17-00-1-0353

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9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSORING / MONITORING AGENCY REPORT NUMBER
U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012	

11. SUPPLEMENTARY NOTES

Original contains color plates; All DTIC reproductions will be in black and white

12a. DISTRIBUTION / AVAILABILITY STATEMENT	12b. DISTRIBUTION CODE
Approved for Public Release; Distribution Unlimited	

13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*

Several studies have suggested an association between low penetrant alleles and breast cancer risk. Although the contribution of low penetrant alleles to the individual risk is relatively small, they can contribute to a large proportion of breast cancer cases in the population. In this study we took the candidate gene approach to study the association of 19 different genetic polymorphisms with breast cancer risk in a population-based sample using a high-throughput genotyping technology. To date, we have completed genotyping 398 cases and 372 population controls for 19 SNPs from several cancer-related molecular pathways. Univariate analysis has shown that XPD cod751 polymorphism is significantly associated with breast cancer risk. None of the remaining 18 SNPs were associated with breast cancer risk individually. Sub-group analysis of the cases has shown that SNPs of ER, XPD, COMT and p27 genes were significantly associated with breast cancer risk in cases with at least a first-degree relative of breast cancer. Cyp17 and MTHFR SNPs were associated with pre-menopausal status, whereas GADD45 and COMT were associated with post-menopausal status. Multivariate analysis of the sample (Logistic Regression Models and Bootstrap analysis) has shown interesting findings regarding the biological interaction between the alleles of cancer-related proteins. The stronger interaction was observed between XPD (DNA repair) and IL-10 (Immune system) SNPs (68%), whereas COMT (Estrogen metabolism) and CyclinD1 interaction shown to be 61% with the bootstrap analysis. The approach used in this study has discovered novel biological interactions between different cancer pathways in the context of breast cancer predisposition. Future studies focusing on systematic selection of functional SNPs and the investigation of their interaction in a larger and homogeneous subset of samples will provide basis for the polygenic model of breast cancer.

14. SUBJECT TERMS		15. NUMBER OF PAGES 54
Cancer genetics, predisposition, SNP, polymorphism, SNParray, Taqman, low penetrating alleles		
		16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited

NSN 7540-01-280-5500

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OVERVIEW

It has long been hypothesized that genetic variation is responsible for observed differences in cancer risk and susceptibility amongst the human population. Mutant alleles of dominant highly penetrant breast cancer genes, including BRCA1 and BRCA2 (1-3), do not occur frequently, and hence account for only a small proportion of breast cancer cases.On the other hand, several studies have suggested an association between low penetrant alleles and breast cancer risk. Although the contribution of low penetrant alleles to the individual breast cancer risk is relatively small, they can contribute to a large proportion of breast cancer cases in the population because the risk-conferring alleles of these genes are common.

Identification and cloning of low penetrant alleles that increase the risk of breast cancer is challenging because the association methods for such studies require large populations to achieve meaningful statistical analysis and very dense genetic maps to facilitate genome-wide genotyping (4,5). At present, the candidate gene approach remains the most logical and practical strategy to identify these risk enhancing, low penetrant variants or single nucleotide polymorphisms (SNPs). Until now, a major obstacle with investigating the risk associated with multiple candidate genes has been a lack of technology for large-scale genotyping of large populations. Consequently, many studies have focused efforts on only 1 or 2 genetic polymorphisms, and even in these cases the analysis was only limited to relatively small sample sizes. In the context of the ideas program, we exploited the high throughput power of SNP genotyping technologies and a well defined, representative population-based sample containing a large number of subjects. We have selected genetic polymorphisms in genes involved in different aspects of carcinogenesis (7-41). For example, cell cycle regulatory genes such as CDK-inhibitors, and cyclins; carcinogen metabolizing enzymes such as CYPs, GSTs and NATs; immune system genes such as interluekins and TNF; and genes involved in other pathways involved in cancer (e.g. p53, PTEN, XPD-DNA repair gene). We have access to the Ontario Familial Breast Cancer Registry (OFBCR), which is the largest population based breast cancer registry in Canada.

The main objective of the proposed work is to identify low penetrant, yet commonly occurring, genetic polymorphisms, which contribute to the risk of developing breast cancer. Furthermore, this approach has the potential to identify novel genetic factors associated with breast cancer risk, which may result in the development of innovative therapies, and a fuller understanding of genetic variation in response to therapy. This will lead to a more complex analysis of gene-gene and gene-environment interactions than is currently possible. Advances in disease etiology will significantly expand our abilities to design strategies for the prevention of breast cancer development and progression

STATEMENT OF WORK

Task 1: Characterization of polymorphic alleles by SSCP, Months 1-8
a. Design of SSCP primers for 32 sites
b. Screen by SSCP analysis for all possible alleles at each locus
c. Sequence the SSCP patterns (appr. 3 per loci) and identify the all possible genotypes

Task 2: Designing of oligonucleotides and sample microarrays, Months 4-8
a. Design different sets of oligonucleotides (perfect matches and mismatches)
b. Customize sample chips for quality control of hybridizations

Task 3: Optimization of the hybridizations using PCR probes, Months 8-16
a. Prepare PCR probes using control specimens
b. Optimize the hybridization conditions
c. Evaluate the accuracy of detection for every polymorphic site using probes with different allelic combinations for each polymorphism. Redesign oligonucleotides and chips in order to increase the quality and accuracy of detection

Task 4 Genotyping of 900 specimens for 32 polymorphisms, Months 16-32
a. Production of microarray chips
b. Preparation of fluorescent labeled PCR probes for each patient
c. Hybridization of chips at optimized conditions
d. Reading and analysis of the chip signals
e. Quality control experiments at different intervals using the control specimens to ensure the reproducibility of results

Task 5. Data and statistical analysis, Months 32-36
a. Repeat and conformation experiments
b. Complete the reading of every slide and prepare the data for statistical analysis
c. Univariate analysis of the data
d. Exploratory multivariate analysis of the data

BODY
A. Strategies and Methodologies Employed

A1. Study Population
In this study we have carried out a case-control analysis using the cases recruited by the population based Ontario Familial Breast Cancer Registry. Breast cancer cases (n=398), under age 55, were sampled from the registry to represent the breast cancer population in general. Healthy population controls (n=372) were recruited randomly similarly to the breast cancer cases. Both cases and controls were matched for ethnicity (Caucasian), gender (Female) and age (Below 55). DNA samples were plated and subjected to genotyping analysis.

A2. SNPs in the Study
We have successfully completed the genotyping of 21 SNPs of genes involved in different pathways of cancer development. In order to understand the breast cancer risk contribution of these SNPs, 19 of them were further analyzed using Univariate and Multivariate logistic regression models. Two of the SNPs, IL1b and p21, were removed from the multivariate models since they were creating high-level noise. Table 1 summarizes the details of the 19 SNPs studied extensively.

A3. SNP Genotyping
In order to assess the specificity for SNP genotyping, we have carried out a validation study where a panel of 150 breast cancer cases and population controls were screened with both SNParray and Taqman methods for all the 21 SNPs in the study. Whereas in over 50% SNPs the results from both methods was concordant, approximately 20% have shown highly discordant results. The remaining SNPs were more comparable to each other. We have repeated a fraction of the discordant results using the two methods complemented by direct sequencing. The differences in results between two methods arouse from poor signal intensity and high background content. Our validation study has shown that with our current setup, Taqman method has provided more reproducible and reliable genotyping results compared to SNParrays. Within the task and the budget of this proposal we have established a high-throughput SNP genotyping platform and carried out extensive method validation. The details of the methods were described previously in the annual report.

Using the 5'nuclease (TaqMan) method (42), we have genotyped 398 breast cancer cases and 372 population controls. Approximately 25% of all cases and controls were genotyped by using both Taqman and SNParrays. Each 96-well micro plate included multiple numbers of cell line DNA specimens representing all possible genotypes of each SNP screened. Furthermore, each micro-plate was designed to contain 10% repeat sample for validation purposes.

A4. Statistical Analysis
We sought evidence of association between each of the 19 SNPs and breast cancer in a multi-step process

A4a. Risk Contribution by Individual SNPs
At the first stage, we calculated crude allele and genotype frequencies for each individual polymorphism. The association between the case-control status and each individual SNP was measured by the OR (odds ratio) and its corresponding 95% confidence interval, estimated using unconditional logistic regression. All analyses were performed using additive, dominant and recessive effect for each polymorphism. In the dominant model, both the heterozygous variant and the rare homozygous variant were combined. In the

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recessive model, the variant was defined as only the rare homozygous genotype and in the additive model both rare homozygous and heterozygous variant effects were estimated. In all analyses, the common homozygote genotype in control population was defined as the reference category. The likelihood ratio test was used to test the effect of each SNP at the nominal 5% significance level. Each SNP were analyzed in a) all cases and controls, b) based on the menopausal status, and c) based on the presence and the absence of family history of breast cancer. Among all the SNPs studied, **XPD Lys751Gln** SNP (DNA repair) was the only one that shown an overall statistically significant association with breast cancer risk (Table 2). Interestingly we have shown that ER, XPD, COMT, and P27 genes have shown significant associations with breast cancer risk in cases with first degree relatives with breast cancer (Table 2). Moreover, Cyp17 and MTHFR SNPs, and COMT and GADD45 SNPs have shown breast cancer risk associations in postmenopausal and pre-menopausal women, respectively (Table 2). None of the SNPs has shown association in cases where there was no first degree relative with breast cancer.

A4b. Risk Contribution by SNP-SNP Interaction

At the second stage, two-way interactions were investigated using multivariable logistic models. More specifically, we tested all SNP-SNP interactions and all SNP by age interactions. We assumed a multiplicative interaction effect on the logit scale. Statistically significant interactions were selected using a forward stepwise selection procedure. The model included all SNP and age as main effects and then search for the most significant candidate interactions to enter into the model based on the score statistics at the 5% level. Backward elimination of variables was based on the likelihood ratio test using the level of 5%. Forward stepwise selection procedure has proven to be efficient in assessing interaction effects as compared to backward elimination when testing multiple interactions. First, it is more time efficient and second, when using backward elimination, a relative large number of predictor variables may increase the risk of complete separation of the two outcome groups, which would yield important numerical problems to estimate the model parameters **(43)**. The stepwise procedure selected 14 significant two-way interactions out of the 190 possible candidates at the 5% entry level **(Table3)**. Because the mode of transmission is uncertain for most of the SNPs considered, we performed these tests on the additive effects only. Among these 14 selected interactions, 5 of them were also statistically significant with the likelihood ratio test at the 5% level *(**Table 3**). Because the large number of interactions analyzed could lead to a high number of false positive findings, we validated our results using bootstrap re-sampling procedures. This statistical method selects random samples of size n with replacement from the original data. Repeating the sampling procedure a large number of times provides information on the variability and validity of the parameter estimate and model selection. Figure 1 demonstrates the statistically significant interactions (validated by bootstrap analysis)

B. Results and Discussion
B1. Main Effect
Long term studies regarding the link between SNPs and genetic diseases have shown that the individual effect of common SNPs are low. The relationship between SNPs and breast cancer risk has been investigated over a decade. A great majority of such studies focus on individual association of one or more SNPs with breast cancer risk. Also, a major fraction of these studies do not have power for making solid conclusions. A few of these studies involve the investigation of several SNP candidates from the same cancer pathway without considering their additive effect on the breast cancer risk. Currently, about 20 SNPs from various cancer pathways have already shown to incrementally

contribute to breast cancer risk (44-60). These risk-conferring SNPs have been found in several cancer pathway genes including estrogen-carcinogen metabolism, DNA repair, cell cycle and others. However, only a few of the studies were able to show overall association with breast cancer risk, and the relative risks obtained in these studies vary between 1.3 -3.0. Most of these studies show modification of breast cancer risk in subgroup analysis such as pre-menopausal or post-menopausal women, or young or old women, yet some others, especially estrogen and carcinogen metabolism genes, show significant results when interaction with environmental factors are considered. Sub-group analysis (relatively more homogenous sample) based on other important risk factors are important to detect increased breast cancer risk, however, such analysis usually lack power due to the partitioning of the sample size, and the lack of power.

Our sample consists of 398 cases and 372 controls, sampled from population based breast cancer registry. All of the cases and controls are Caucasian women under age 55. In the analysis of 19 individual SNPs, XPD Lys751Gln SNP was the only one that showed a significant association with breast cancer risk. None of the other 18 SNPs has shown a significant overall association with breast cancer risk.

B2. SNP-SNP Interactions and Breast Cancer Risk

Our main goal in this study is to study the SNP-SNP interactions between various cancer pathway SNPs. Because of the sample size we have only focused on 2-by-2 interactions. Forward model including 19 SNPs and age as a variable has provided 14 statistically significant SNP-SNP interactions. These results were validated using 1000 boostrap analysis of the forward model. Using this approach we have shown the percentage of each interaction coming up significantly (Table 3). Nine SNPs were confirmed to be involved in 2-by-2 interactions that were detected more than 50% at a time by boostrap analysis (Table 3, Figure 1). Among these interactions XPD-IL10 (68%), cyclinD1-Age (64%), COMT-CylinD1 (61%) was among the most likely interactions. XPDs interaction (top interaction in boostrap analysis) may be influenced by the fact that the XPD Lys751Gln individually has a main effect however in the case of cyclinD1-COMT (61%), none of the SNPs had shown main effect in the whole group (manuscript submitted).

Significant interactions between SNPs, which did not have main effects, are a novelty of our study. Most of the studies, through years, have investigated the main effects of such SNPs and categorized them as not associated, thus not important to breast cancer risk. However, our study showed that this is not the case and that SNPs without main effects may interact and confer an increased risk of breast cancer. Interaction between SNPs have also previously reported in several studies, majorly focusing on carcinogen metabolism genes like GSTM1, GSTT1, GSTP1, GSTM3 and CYP genes (61-67). These reports support our findings regarding an additive effect of SNPs to breast cancer risk, although these studies only limited to a single pathway interactions.

B3. Systems Biology and Protein Networking

Our study strongly suggests a cross talk between the proteins of different cancer pathways in the context of breast cancer predisposition. In this study we have provided a SNP-based polygenic model from breast cancer risk. The study implicates that the small effect of individual SNPs can be added to result in a more dramatic increase in risk. This study indicates a proof-of-principle for a SNP-based polygenic model and suggests the application of this approach to other SNPs and diseases in a systematic manner. Our recommendation for a future study includes systematic selection of common SNPs to be studied on the basis of its function and its relevance to disease, and hypothesis driven selection of relevant SNP subsets for statistical analysis on the basis of their biological relevance to the function and the disease.

C. Conclusion

Our focus in this study has been to build a biological-knowledge based polygenic model for breast cancer predisposition. From our small number of SNP pool we have shown significant statistical and thus biological interaction between several genes/SNPs from various cancer pathways. Our immediate task is to apply this strategy to a larger sample, with an aim to investigate more complex interactions (3-by-3, 4-by-4 etc).

This line of research has a potential to identify important cross talk between the members of the cancer pathways in the context of the disease. This study does not only provide light for the analysis of the polygenic nature of breast cancer, but also provide important information regarding how cell functions during the disease state. We believe that these and other interactions in breast cancer will one day be identified and used in the clinics to identify individuals at increased risk of breast cancer. This research will reach to a much greater portion of the breast cancer patients in the population compared to carriers of single-gene high penetrant mutations.

D. Key Research Accomplishments

We have accomplished the tasks proposed in the Statement of Work by

- Genotyping and validation of 398 breast cancer cases and 372 population controls for 21 SNPs
- Statistical analysis to evaluate the main effects of the individual SNPs to breast cancer risk. XPD Lys751Gln was shown to be associated with breast cancer risk.
- Statistical modeling to study the risk contributed by 2-way SNP interactions. Several interactions involving SNPs of different cancer pathways have been discovered in the context of breast cancer.
- This study provided evidence for the multigenic model of breast cancer involving SNP interactions.

G. Reportable Outcomes

G1. Presentations

Venus Onay, Julia Knight, and Hilmi Ozcelik, "Microarray Technology to Study the Role of Candidate SNPs in Breast Cancer Risk" 3rd International Meeting on Single Nucleotide Polymorphism and Complex Genome Analysis, 8th-11th September 2000, Taos, New Mexico, USA..

Venus Onay, Julia Knight, and Hilmi Ozcelik, "Identifying the Role of SNPs in Breast Cancer Risk Using Microarray Technology." Oncogenomics Conference, 25-27 January 2001, Tucson, Arizona, USA.

Venus Onay, Julia Knight, and Hilmi Ozcelik, "Candidate SNP Analysis in the Study of Breast Cancer Risk Using SNParrays" 93rd Annual Meeting of AACR, April 6-10, 2002, San Francisco, California, USA.

Venus Onay, Julia Knight, and Hilmi Ozcelik, "Candidate SNP Analysis in the Study of Breast Cancer Risk Using SNParrays" Controversies in the Etiology, Detection and Treatment of Breast Cancer:2002, June 13-14, 2002, Toronto, Ontario, Canada.

Venus Onay, Julia Knight, Sean Wells, and Hilmi Ozcelik, "Candidate SNP Analysis in the Study of Breast Cancer Risk Using SNParrays" The 4th Era of Hope Meeting for the Department of Defense Breast Cancer Research Program, September 25-28, 2002, Orlando, Florida, USA.

U. Venus Onay, Julia Knight, Sean Wells, Hong Li, and Hilmi Ozcelik, "Investigating the Role of 24 Variants from Major Cancer Related Pathways in Breast Cancer,"

Cancer Family Registries of Breast and Colon Cancer, Scientific Meeting, January 15-17, 2003, Waikoloa, Hawaii.

U. Venus Onay, Julia Knight, Sean Wells, Hong Li, and Hilmi Ozcelik, "Investigating the Role of 24 Variants from Major Cancer Related Pathways in Breast Cancer," AACR International Conference on Molecular and Genetic Epidemiology of Cancer; January 18-23, 2003, Waikoloa, Hawaii.

U. Venus Onay, Julia Knight, Sean Wells, Ellen Shi, Hong Li, and Hilmi Ozcelik, "Investigating the Cross-Talk Between Cancer-Related Pathways Based on Molecular Epidemiological Studies" *Program and abstracts of SNPs, Haplotypes, and Cancer: Applications in Molecular Epidemiology*, September 13-17, 2003, Key Biscane, Florida, USA.

U. Venus Onay, Julia Knight, and Hilmi Ozcelik, " A Biological Interaction between DNA Repair and immune System SNPs in Breast Cancer Predisposition" *Proceedings 95th Annual Meeting of AACR, Mach27-31,2004*, Orlando, Florida, USA

G2. Publications:

U. Venus Onay, Julia A. Knight, Sean Wells, Hong Li, Ellen Shi, Irene L. Andrulis, Laurent Briollais, Hilmi Ozcelik, "Breast Cancer Risk Conferred by Cross-Talk between Commonly Occurring Polymorphisms of COMT and Cyclin D1," submitted, *Cancer Epidemiol Biomarkers Prev.*

Onay UV, Knight JA, Wells S, Hong L, Andrulis IL, Briollais L, Ozcelik H. Interaction between the SNPs of Major Cancer Pathways: A Polygenic Model for Breast Cancer Predisposition, in preparation.

Onay UV, Figueiredo J, Knight JA, Wells S, Hong L, Andrulis IL, Briollais L, Ozcelik H., "A DNA repair SNP, XPD 751, and Breast Cancer Risk" (in preperation).

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Table1. A summary of the SNPs studied.

Gene	SNP common name	Nt. Change	Accession number	Minor Allele frequencies	Function from literature	Ref	Breast cancer associations	Ref
XPD	Lys751Gln	A35931G	L47234.1	C:27%	Gln allele has decreased DNA repair capacity	68-71	not overall, (interaction with environment)	72
ER	Ser10Ser	T270C	M69297.1	T:48%	no function studied	--	Yes (subgroup)	73
MTHFR	Ala222Val	C677T	U09806.2	T:37%	Val allele is associated with reduced enzyme activity.	74	yes and no	75-79
BARD1	Pro24Ser	C243T	AF038034.1	not available	no functional studies done	--	not studied	--
MMP1	1G(-1607)2G	168(ins/delG)	AJ002550.1	G:39%	insG allele creates an Ets binding site and cooperates with nearby AP-1 binding site to increase transcription	80,81	not studied	--
CyclinD1	Pro241Pro	A6962G	11436818	A:48%	A allele-no nuclear export and cytoplasmic degradation	82-87	no assoc.	88
p27	Val109Gly	G326T	X84849.1	G:28%	no functional studies done		not studied	--
GADD45	C(IVS3+168)T	C3812T	L24498.1	not available	no functional studies done	--	not studied	--
PTEN	(IVS4+109)ins/delACTAA	9343 (ins/delACTAA)	AF143314.1	not available	no allelic function change	89	associated with earlier age of breast cancer onset in variant homozygotes	90
ER	Pro325Pro	C1267G	M12674.1	G:27%	no function studied	--	yes (lymph node metastasis)	73,91
CYP17	CAC-box	C518T	M31146.1	A:47%	associated with increased serum estradiol	92	yes (majority in subgroups) and no	93-102
COMT	Met108/158Val	G1947A	Z26491.1	A:47%	Met allele is thermolabile and has lower enzymatic activity	103,104	controversial	105-114
GSTM3		4595 (3bp ins/del)	AF043105.1	not available	the del allele creates a recongnition site for a YY1 transcriptional regulatory factor	115	yes (subgroup)	116
GSTP1	Ile105Val	A1568G	X08094.1	G:31%	Val allele is associated with reduced enzyme activity towards alkylating agents.	117	no and yes	118-124
G-CSF	Leu185Leu	G1780A	X03656.1	A:47%	no functional studies done	--	not studied	--
IL13	Arg130Gln	A2814G	L13029.1	A:15%	no functional studies done	--	not studied	--
TNFA	G(-308)A	G89A	U42625.1	not available	(increased transcriptional activity) and (no functional change)	125-128	no	129
IL1A	Ala114Ser	T6282G	X03833.1	T:35%	no functional studies done but in linkage with (-899) SNP which might have a function	130	not studied	--
IL10	G(-1082)A	G2934A	X78437.1	G:36%	G allele associated with increased expression	131-133	yes	134

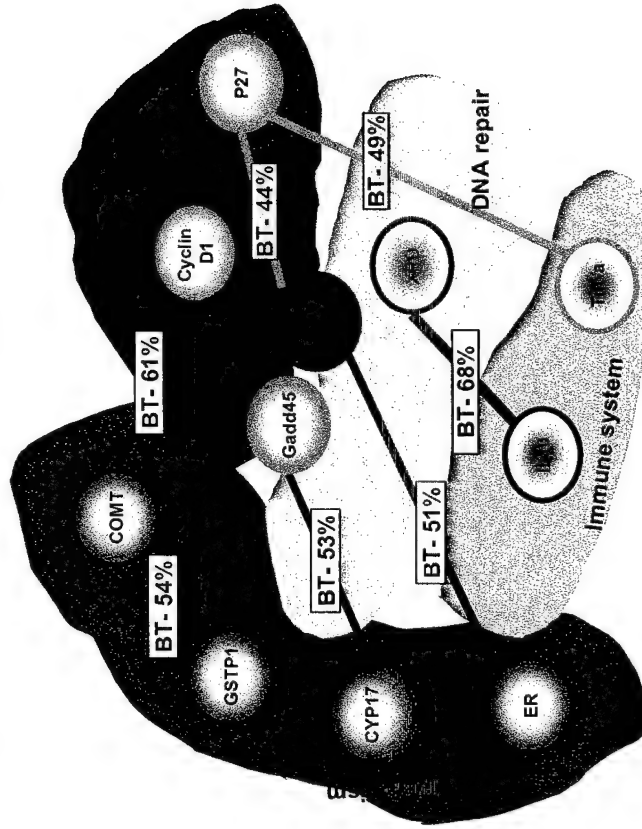**Table 2:** SNPs that showed overall or subgroup association with breast cancer risk.

Gene	SNP	Cases n=	Controls n=	Sample Group	Associated allele	OR (95% CI)	Pathway
XPD	Lys751Gln	398	372	All	Gln/Gln or Lys/Gln	1.4 (1.01-1.80)	DNA repair
Cyp17	C518T	98	110	Post-menopausal	CC or CT	1.9 (1.13-3.50)	Estrogen metabolism
MTHFR	Ala222Val	98	110	Post-menopausal	Val/Val	2.7 (1.13-6.61)	Metabolism
GADD45	C(IVS3+168)T	241	246	Pre-menopausal	CC or CT	0.7 (0.48-0.98)	Cell cycle, DNA repair
COMT	Met108/158Val	241	246	Pre-menopausal	Val/Val or Met/Val	1.7 (1.1-2.55)	Estrogen metabolism
COMT	Met108/158Val	52	17	Family History	Met/Val	2.622 (1.018-6.751)	Estrogen metabolism
ER	Ser10Ser	64	21	Family History	CT or CC	2.667 (1.117-6.367)	Estrogen metabolism
p27	Val109Gly	34	8	Family History	Val/Gly	2.511 (1.011-6.239)	Cell cycle
XPD	Lys751Gln	52	15	Family History	Gln/Gln or Lys/Gln	2.476 (1.099-5.577)	DNA repair

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Table 3. Interactions found with multivariate logistic regression using forward stepwise selection model. *P*- values assigned for each interaction and for the likelihood ratio test, both at 95% significance level is added. The interactions that are found significant in both are bolded.

A. Interaction	p value	LRT test p-values	Bootstrap (forward model)
	(95% significance)		
XPD and IL10	**0.0349**	**p=0.01**	**68%**
Age and CyclinD1	**0.0296**	**p=0.011**	**64%**
COMT and cyclinD1	**0.0101**	**p=0.035**	**61%**
GSTP1 and COMT	**0.0355**	**p=0.052**	**54%**
CYP17 and GADD45	0.0243	p=0.13	53%
Bard1 and ER-cd325	0.0393	p=0.077	51%
TNFA and p27	**0.0164**	**p=0.026**	**49%**
Age and XPD	0.0228	p=0.063	46%
Bard1 and p27	**0.0205**	**p=0.016**	**44%**
Bard1 and XPD	0.0241	p=0.053	36%
ER-cd10 and ER-cd325	0.0282	p=0.11	30%
IL13 and IL10	0.0112	p=0.15	29%
IL13 and COMT	0.0155	p=0.195	28%
IL1A and GSTM3	0.0262	p=0.41	27%
tnfA and COMT	nonsignificant	**p=0.02**	**27%**

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Figure 1: Interaction between SNPs of cancer genes determined by Logistic Regression Models and Bootstrap analysis.

Cross-Talk Between Proteins and Pathways

(Statistical Protein Networks of Breast Cancer)

Breast Cancer Risk Conferred by Cross-Talk between Commonly Occurring Polymorphisms of COMT and Cyclin D1

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Running Title: Interaction between COMT and cyclin D1 in Breast Cancer

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Abstract

Estrogens are crucial in cell growth and proliferation, and can be tumorigenic. Catechol-O-methyl transferase (COMT) detoxifies the catechol estrogen metabolites. A single nucleotide polymorphism (SNP), Met108/158Val, in COMT gene has been previously associated with cancer risk. The enzymatic activity of COMTMet^L allele has been suggested to be 3 to 4-fold less compared to the COMTVal^H allele. Several studies have also shown the relationship between estrogen and cell cycle progression through activation of cyclin D1 transcription. Another SNP in cyclin D1 (A allele of Pro241Pro) has also been hypothesized to produce a more stable protein compared to the G allele. Here, we describe the interaction between the SNPs of COMT and Cylin D1, in the context of increased breast cancer risk, in a case-control study of 398 Caucasian breast cancer cases and 372 Caucasian population controls. Cyclin D1 Pro241Pro SNP did not show any significant association with breast cancer risk (OR:1.265, 95%CI:0.924-1.733), whereas an increased breast cancer risk was associated with the COMTVal^H allele only in pre-menopausal women (OR:1.68, 95%CI:1.10-2.55, p=0.016). Although the main effect of individual SNPs were not strong, their interaction contributes more considerably to increased breast cancer risk (p=0.035). The greatest magnitude of increased in risk was observed in pre-menopausal women by the interaction of at least one COMTVal^H allele, and with at least one cyclin D1^A allele (OR:3.49, 95%CI:1.59-7.68, p=0.0019).

This study demonstrates an example for gene-gene interaction between common, low penetrant, alleles in the population, which suggest a polygenic model for the genetics of breast cancer.

Introduction

Estrogens demonstrate diverse effects in humans and have a role in breast cancer development. Estrogen exerts its effect by simultaneously stimulating the transcription of genes, via the estrogen receptor, necessary for cell proliferation and by causing DNA damage via their catechol estrogen metabolites (1, 2). Involvement of estrogens in tumorigenesis is based on the consensus that cell division plays an important role in cancer development. Reproductive factors that increase the mitotic activity in the breast epithelium also increase cancer risk. It has been suggested that the risk of breast cancer could be determined by cumulative exposure of breast tissue to estrogens during reproductive life (3). Supporting this, epidemiological studies have shown that early menarche, late first-full term pregnancy, and late menopause are the most significant risk factors for breast cancer development (4, 5).

Estrogens are eliminated from the body by metabolic conversion to hormonally less active water-soluble metabolites that are excreted in the urine. The two major estrogens, 17B-estradiol (E2) and estrone (E1), are oxidized to the 2-OH and 4-OH catechol estrogens and 16-a hydroxyestrogen by Cyp1A1 and Cyp1B1 enzymes (6, 7). The products of the phase I enzymes are extremely toxic metabolites, which are congujated by several phase II enzymes like sulfotransferases, glucuronosyltransferases and estrogenacyltransferases (8). Catechol estrogens are substrates for the phase II enzyme, catechol-O-methyl transferase (COMT), which catalyzes their conversion into biologically non-hazardous methoxyestrogens. Most detoxification happens in the liver, but it takes place in peripheral tissues as well, including breast (8). Several studies have shown that some polymorphisms of

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metabolism genes, including COMT, contribute to breast cancer risk. However, its contribution to breast cancer risk is controversial.

Two isoforms of COMT, S-COMT and MB-COMT, have been found in cytosol, and in the cytoplasmic portion of endoplasmic reticulum, respectively. It is constitutively expressed mainly in brain, liver and kidney, but also in peripheral tissue, including the epithelial cells in the ducti and lobuli of normal mammary tissue. COMT expression is elevated in tumors compared to normal mammary tissue (9). COMT activity varies among individuals, and lower activity is associated with low thermal stability (10, 11). A SNP in 108/158th amino acid in the protein sequence results in two different alleles of COMT (A to G change at position 1947 in accession number Z26491.1), COMTMet[L] and COMTVal[H]. In the Caucasian population the allele frequencies are 0.47 and 0.53, respectively[1]. It has been suggested that the enzymatic activity of COMTMet[L] may be 3 to 4-fold less active compared to COMTVal[H] (12, 13). Association studies addressing the contribution of COMT alleles to breast cancer risk have been inconsistent. Increased breast cancer risk has been shown to be associated with both low and high activity alleles of COMT mainly in subgroup analyses in Caucasian (14-18) and Asian populations (19, 20). Some other studies failed to show any significant association between COMT activity alone and breast cancer risk (21-23).

Steroid hormones like estrogen are major regulators of cell cycle progression in breast cancer cells (24). Several studies have shown the relationship between estrogen and cell cycle progression through activation of cyclin D1 transcription (25, 26). Cyclin D1 is the key regulator of transition of the cell from G1 to its proliferative S phase. Cyclin D1 accumulates and activates CDK4/6 in response to mitogenic growth factors in

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early to mid G1 phase, and initiates the transcription of transcription factors required in the subsequent S phase. Excess accumulation of cyclin D1 in a cell due to either amplification of cyclin D1 gene or over-expression of its protein product has been frequently found in various cancers, including breast cancer (27).

With respect to the genetic variants of cyclin D1, it is suggested that a G to A substitution at position 6962 (accession number 11436818) (Pro241Pro) in exon 4 produces two alternatively spliced forms of transcript. Splicing isoform cyclin D1b produced by the cyclin D1[A] allele lacks exon 5 (28). This last exon of cyclin D1 contains a rapid protein degradation motif (PEST), and the protein product of the cyclin D1[A] allele is hypothesized to be more stable compared to the product of cyclin D1[G] allele (28). It also has been observed that splicing form lacking exon 5, thus lacking a phosphorylated Thr residue (Thr286), is unable to be transported to cytoplasm and unable to be ubiquitinated/degraded (29,30,31) and is a nuclear oncogene (32). A number of association studies have also identified the cyclin D1[A] as a risk allele for colorectal (33,34), lung (35), prostate (36) and esophageal (37) cancers. A breast cancer study however did not show any association of this allele with breast cancer (38). The frequency of the cyclin D1[A] allele in Caucasian population is 0.48[2].

In an attempt to identify the potential breast cancer risk alleles, we have selected 19 SNPs from genes involved in major cancer related pathways (COMT, Cyclin D1, ERa, Cyp17, MHTFR, GADD45, MMP1, TNFA, G-CSF, IL1a, IL10, IL13, XPD, BARD1, p27, PTEN, GSTP1 and GSTM3). Univariate and multivariate analyses revealed several statistically significant individual associations and gene-gene interactions in the context of breast cancer. In this study, we describe the interaction between cyclin D1

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Pro241Pro and COMT Met108/158Val SNPs associated with increased breast cancer. To our knowledge this interaction has not been described previously. This study reveals a novel biological interaction in breast cancer between the actions of COMT and cyclin D1 alleles.

Materials and Methods

Subject populations

A case control study was conducted making use of the Ontario Familial Breast Cancer Registry (OFBCR) a participating site in the US NIH Breast Cancer Family Registry. The OFBCR has been described more fully elsewhere (39). Cases of invasive breast cancer, pathologically confirmed, and diagnosed between 1996 and 1998 in the province of Ontario were identified from the Ontario Cancer Registry (OCR) within approximately six months of diagnosis in most cases. All female cases under 55, a random sample of female cases aged 55 to 69, and all male cases under age 80 were identified. However, the current study was restricted to women under 55 for two reasons. The number of older women available for inclusion was limited and it was hypothesized that genetic alterations would play a stronger role in younger women. Physicians were contacted to obtain permission to contact patients and permission was granted for 91% of cases (7668 of 8453). Patients who could be contacted were then mailed a cancer family history questionnaire and 65% (4957) completed it. All respondents who met a defined set of genetic risk criteria and a random sample of 25% of those not meeting criteria were selected to continue to participate in the OFBCR (n= 2580). This participation included completing a mailed risk factor questionnaire

(completed by 72% of all eligible, n=1848) and providing a blood sample (provided by 62% of all eligible, n=1601). For the current study, we restricted the sample to those who self-identified as Caucasian only and had provided blood. Also, because of the 25% random sample of those who did not meet genetic risk criteria, we randomly sampled 25% of those who did meet genetic risk criteria in order to create a more generally representative sample of cases. There were 459 Caucasian cases with blood available selected. After exclusion of those with insufficient DNA or who could not be genotyped for other reasons, 398 cases were genotyped, 347 of whom also had risk factor questionnaire data available (246 premenopausal, 98 postmenopausal, and 3 unknown).

Controls were identified by calling randomly selected residential telephone numbers from across the province of Ontario and were frequency-matched to all female OFBCR cases by ethnicity and 5-year age group. The number of telephone numbers was 14,653, but 1101 (8%) were invalid and no contact could be made for 841 (6%). Of the 12,711 households contacted, 7829 (62%) did not have an eligible individual. No information on eligibility was provided for 2194 (17%) households. Of the 2688 eligible individuals identified on the telephone, 1726 (64%) completed the mailed risk factor questionnaire. Six hundred and seventy-six women were asked to provide a blood sample, randomly selected from those under 55 who had agreed to be approached about blood sampling (75% agreed), and blood samples were obtained from 419 (62%). Individuals who were not Caucasian were excluded from the analysis as were those with insufficient DNA or those subsequently found to be ineligible because of age. This

left 372 controls (240 premenopausal, 110 postmenopausal, and 12 unknown) with genotypes available.

Molecular Genotyping:

Both cyclin D1 and COMT SNPs were analysed by TaqMan 5'nuclease assay (40). Oligonucleotide primers and the dual labelled allele specific probes were designed using PrimerExpress version 2.0 (PE Biosystems). Positions of primers for COMTMet108/158Val SNP are [(5'CCCAGCGGATGGTGGAT3') and (5'CCCTTTTTCCAGGTCTGACAAC3')] and probes [(5'^{6FAM}CACCTTGTCCTT CA$\underline{C}$GCCAGCGA^{BHQ1}3') and (5'^{TET}CACACCTTGTCCTTCA$\underline{T}$GCCAGCGA^{BHQ1}-3')] in accession number Z26491.1 are (1921-1937), (1975-1997), (1939-1961) and (1937-1961), respectively. Locations of primers for cyclinD1 Pro241Pro SNP are [(5'CTGAGGAGCCCCAACAACTTC3') and (5'ACTAGGTGTCTCCCCCTGTAAGC3')] and probes [(5'^{6FAM}CCTCACTTAC$\underline{C}$GGGTCA$^{MGB-NFQ}$3') and (5'^{VIC}CCCTCACTTAC $\underline{T}$GGGTCA$^{MGB-NFQ}$-3')] in accession number 11436818 are (6878-6908), (6999-7021), (6956-6972) and (6956-6973), respectively.

A number of DNA samples were sequenced for each SNP beforehand, to identify genotyping controls in each experiment. Amplification reactions were performed in 96 well plates (AXYGEN). Each plate contained four control DNAs for each possible genotype. Genomic DNA (10ng) was amplified in a total volume of 10 μl in the presence of 100 μM of each of the dNTPs, 3 pmoles of each of the appropriate primers, 2 pmoles of each of the corresponding dual labelled probes, and 0.025 Unit of Platinum Taq DNA Polymerase (InVitrogen). The Mg concentration was 3 mM for COMT and 4

mM for cyclin D1. A homemade PCR buffer was used in 1X concentration in the reactions. PCR cycling conditions consisted of 40 cycles of 94^0C for 15 sec, X^0C for 15 sec and 72^0C for 15 sec, X being 60 for COMT and 58 for cyclin D1 SNPs. The reactions were analyzed by ABI PRISM 7900HT Sequence Detection System (version 2.0). For validation purposes, 10% of the study population was randomly selected and re-genotyped and their genotypes were confirmed.

Statistical Analysis

The association between the case-control status and 19 individual SNPs were measured by the OR (odds ratio) estimated using unconditional logistic regression. The corresponding 95% confidence intervals were also calculated. All statistical analyses were carried out separately in the overall sample and in pre-menopausal women only. OR and 95% CI (confidence intervals) were calculated for each SNP genotype, and under two genetic models (dominant and recessive). In the dominant model, both the heterozygous variant and the homozygous variant were combined, whereas in the recessive model, the variant was defined as only the homozygous genotype. At the first stage, univariate analyses were performed for each individual SNP. The likelihood ratio test was used to test the significance of each SNP at the 5% level. At the second stage, two-way interactions were investigated using multivariable logistic models. More specifically, we tested all SNP-SNP interactions and all SNP by age interactions. Without loss of generality, we assumed a multiplicative interaction effect on the logit scale. Statistically significant interactions were selected using a forward stepwise selection procedure. The model included all SNP and age as main effects and then

search for the most significant candidate interactions to enter into the model based on the score statistics at the 5% level. Backward elimination of variables was based on the likelihood ratio test using the level of 5%. Forward stepwise selection procedure has proven to be efficient in assessing interaction effects as compared to backward elimination when testing multiple interactions. First, it is more time efficient and second, when using backward elimination, a relative large number of predictor variables may increase the risk of complete separation of the two outcome groups, which would yield important numerical problems to estimate the model parameters (41). The stepwise procedure selected 14 significant two-way interactions out of the 190 possible candidates at the 5% entry level. Among these 14 selected interactions, 6 of them were also statistically significant with the likelihood ratio test at the 5% level. The interaction between COMT and cyclinD1 was the most significant one with an associated p-value of 0.035. To assess the uncertainty of model selection, we used bootstrap re-sampling techniques. Out of 600 bootstrap samples, the interaction between COMT and CyclinD1 was selected 363 (60.5 %) times at the 5% entry level. All statistical analyses were performed with the software SAS. The reference homozygote genotype was selected as the one with the highest frequency in the control population, both in univariate and in multivariate analyses.

Results

Both COMT and cyclin D1 SNPs occur very frequently in the general population. In 372 controls studied, 48.92% were heterozygous for COMTVal^{H}Met^L, 22.85% were

homozygous for COMTVal^H, and 28.23% for COMTMet^L. We found 46.51% of the control population heterozygous for cyclin D1^{AG}, and 22.85% and 30.65% were homozygous for cyclin D1^A and cyclin D1^G alleles respectively.

Analyses in the whole sample have shown that high enzymatic activity COMTVal^H allele contributed to breast cancer risk with a borderline significance in a dominant model (OR:1.33, 95%CI:0.96-1.84, p=0.08) (Table 1). There was a stronger association between the same allele and breast cancer risk in pre-menopausal women (OR:1.68, 95%CI:1.10-2.55, p:0.016). Analysis of the individual cyclin D1 polymorphism did not show any significant association with breast cancer risk (OR:1.265, 95%CI:0.924-1.733, p:0.14) (Table 1) in the whole sample or pre-menopausal subgroup.

Although the effects of individual gene polymorphisms were not strongly associated with breast cancer risk, their interaction (p=0.035) contributes more considerably to increased breast cancer risk. The interactions, assuming a dominant model for both polymorphisms, are shown in Table 2. The reference category consists of those who are homozygous for both the low activity COMT allele and the cyclin D1 G allele. The comparison groups are those who carry one or the other or both variants. All of the genotype groups in the whole data set and in premenopausal women revealed increased breast cancer risk compared to the reference genotype (COMTMet^L/Met^L|cyclinD1^{GG}), with a clear trend in this increase from the first to last group (Figure 1). The greatest magnitude of increase in risk was observed in pre-menopausal women, in the groups considering a dominant effect for both SNPs (OR:3.49, 95%CI:1.59-7.68, p=0.0019) .

Discussion

In this study we have shown a statistically significant association between the COMT-Val^H allele and breast cancer risk in Caucasian pre-menopausal women. This is a functional polymorphism that has been shown in two independent studies, where recombinant forms of two COMT variants were expressed in order to compare catalytic activities of both alleles. The COMTMet^L allele was found to have approximately 60-80% lower catalytic activity compared to COMTVal^H allele (42,43).

Several groups have studied the association of COMT with breast cancer risk; however their findings are somewhat ambiguous. In agreement with our findings, three reports studying Caucasian populations (with a similar number or more cases than in our study) have demonstrated that the COMTVal^H allele is associated with increased breast cancer risk (16-18). Other studies, in contrast, have shown that the COMTMet^L allele is associated with increased breast cancer risk (15, 19, 20). However, two of these studies were in non-Caucasian populations (19, 20). Therefore, 3 out of 4 studies in Caucasians have identified the COMTVal^H allele as the increased risk allele, although not always significantly. Hong et al (44) also found a significant association between COMTVal^H allele and higher breast density in pre-menopausal women without breast cancer. It has been shown that higher breast density is associated with increased risk of breast cancer (45). The overall conclusion of the review of these studies is that COMT alone is not significantly associated with breast cancer. However, COMT status may modify the risk of breast cancer in concert with other genetic or environmental factors.

Interaction between COMT and cyclin D1 in breast cancer

COMT plays an important role in the metabolism of estrogen, which is a well-known risk factor for breast cancer in women. Epidemiological studies have long shown that the exposure to estrogen during reproductive life has considerable effect in developing breast cancer. Estrogen exerts its biologic effect mainly through initiating the expression of genes necessary for cell proliferation, which is known to induce cancer formation (46). Estrogen is broken-down by Cyp1A1 and Cyp1B1 into catechol estrogens, and 16-a hydroxyestrogen, which are known to cause DNA damage. COMT, however, metabolizes the catechol estrogens further into methoxy estrogens. The 2-OMe-estrogen and 4-OMe-estrogen produced by COMT have recently been shown to act in a negative feedback inhibition of Cyp1A1 and Cyp1B1 in MCF7 cells (47). Methoxy estrogens thus compete with estrogen for binding to Cyp1A1 and Cyp1B1 proteins. In accordance with this evidence, COMT with high enzymatic activity is expected to lead to a reduction of metabolism of estrogen by CYP enzymes. This suggests the presence of decreased estrogen metabolism and thus increase estrogen levels in the cell/body. This hypothesis is in accordance with our finding regarding the statistically significant association of COMTVal[H] with increased breast cancer risk in pre-menopausal women (Figure 1). In contrast with our hypothesis, a study has shown increased serum estrogen levels in women with at least one COMTMet[L] allele (48). However, the sample size of this study is small (n=36) and it included only peri and postmenopausal women.

More interestingly, breast cancer risk was dramatically increased in the context of interaction of high activity COMTVal[H] and protein stabilizing cyclinD1[A] alleles of these genes (Table 2, Figure 1). Cyclin D1 gene produces an isoform through alternative

splicing (cyclin D1b). The carboxy-terminal of cyclin D1b isoform lacks the PEST protein degradation motif and the Thr 286 amino acid residue within this motif. Phosphorylation of this threonine residue enables nuclear export and ubiquitination/degradation of the protein. The protein product of cyclin D1[A] allele therefore is defective in nuclear export, thus accumulates in the nucleus, during the cell cycle and facilitates cellular transformation. This statistically significant (p=0.035) gene-gene interaction between this cyclin D1b and COMTVal[H], suggests a cross-talk between these two alleles in the context of breast cancer predisposition.

Estrogens have been shown to activate the G1/S transition through both cyclin D1 dependent and independent mechanisms. The balance is maintained between the estrogen levels in the cell and the functionality of the enzymes that metabolize estrogen, including Cyp1A1, Cyp1B1 and COMT. Estrogen regulates cyclin D1 expression in different ways. Estrogen dependent cyclin D1 expression is believed to be regulated via an estrogen-activated classical cytosolic/nuclear estrogen alpha (49,50) or unidentified plasma membrane bound ER activation (51). The role of estrogen in increasing cell proliferation, through nuclear or membrane bound receptors, has been shown extensively (52,53). Recently, it has been demonstrated that estrogen's effect on cyclin D1 transcription is mediated through PI3K/Akt and map kinase pathways (50, 51), both of which are important for cell signalling and cell cycle progression. Moreover, Diehl et al shown that the phosphorylation and nuclear export of the cyclin D1 is performed by GSK3B, activity of which also is controlled by PIK3/Akt kinase. When cultured cells are serum activated the increased activity of PI3K/Akt pathway inhibited GSK3B activity, which in turn could not phosphorylate cyclin D1, and inhibited its nuclear export. The

model describing the importance of the interaction of these two genes is schematically illustrated in Figure 2.

Although being a fairly good size, our sample size is a limitation to this study. Since we need to consider nine different genotype combinations of two SNPs, some genotypes are represented in fairly small numbers. Moreover, there is always a risk of finding a false positive interaction, however we believe our attempt to confirm this interaction by bootstrap method was effective enough by finding this particular interaction almost 61% of all 600 runs.

As a conclusion, we have shown a disadvantageous biological interaction between the two commonly occurring polymorphisms of COMTVal[H] (47.3%) and cyclin D1[A] (46.1%) alleles in the context of breast cancer predisposition. These SNPs, individually, were not associated with breast cancer risk strongly. Our findings suggest that COMT and cyclin D1 alleles interact in the presence of estrogen and initiate the events necessary for cancer progression. Here we propose that the allelic status of individuals with respect to these two genes alters the relative risk of individuals for breast cancer. It is possible that estrogens might induce cancers by changing the rate of the cell division, thus increasing the potential for accumulation of spontaneous mutations. This study demonstrates the importance of molecular epidemiological studies in uncovering interaction in the context of disease. The model described in this study demonstrates the importance of gene-gene interaction between low penetrant alleles, which provides guidance to the understanding of the genetic basis of breast cancer, providing a model for complex diseases in general.

Interaction between COMT and cyclin D1 in breast cancer

Acknowledgements

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Footnotes

[1]http://snp500cancer.nci.nih.gov/snp.cfm?snp_id=4680&ethnic=true&poly_id=COMT-01

[2]http://snp500cancer.nci.nih.gov/snp.cfm?snp_id=603965&ethnic=true&poly_id=CCND1
-02

[3]**Met**^{L}: Low activity methionine allele, **Val**^{H}: High activity valine allele, **SNP:** Single

nucleotide polymorphism, **E1:** Estrone, **E2:** Estradiol, **COMT:** Catechol-O-methyl

transferase, **S-COMT:** Soluble Catechol-O-methyl transferase, **MB-COMT:** Membrane

bound Catechol-O-methyl transferase, **Cyp1A1:** Cytochrome P450 A1, **Cyp1B1:**

Cytochrome P450 B1, **-OH:** hydroxyl.

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Table 1. Individual association analysis for COMT-Met108/158Val and Cyclin D1Pro241Pro SNPs with breast cancer risk. The analyses of the whole group and premenopausal subgroup are included.

COMT-Met108/158Val			
Genotype	**Cases, n (%)**	**Controls, n (%)**	**OR (95% CI)**
All			
Met^L/Met^L	91 (22.86)	105 (28.23)	1
Met^L/Val^H	210 (52.76)	182 (48.92)	1.33 (0.94-1.88)
Val^H/Val^H	97 (24.37)	85 (22.85)	1.32 (0.88-1.97)
Met^L/Val^H or Val^H/Val^H	307 (77.14)	267 (71.77)	1.33 (0.96-1.84)
Pre-menopausal			
Met^L/Met^L	47 (19.5)	71 (28.86)	1
Met^L/Val^H	141 (58.51)	114 (46.34)	**1.87 (1.20-2.91)**
Val^H/Val^H	53 (21.99)	61 (24.8)	1.31 (0.78-2.21)
Met^L/Val^H or Val^H/Val^H	194 (80.5)	175 (71.14)	**1.68 (1.10-2.55)**
CyclinD1-Pro241Pro			
Genotype	**Cases, n (%)**	**Controls, n (%)**	**OR (95% CI)**
All			
GG	103 (25.88)	114 (30.65)	1
AG	196 (49.25)	173 (46.51)	1.25 (0.90-1.75)

Interaction between COMT and cyclin D1 in breast cancer

AA	99 (24.87)	85 (22.85)	1.29 (0.87-1.91)
AG or AA	295 (74.12)	258 (69.35)	1.27 (0.92-1.73)
Pre-menopausal			
GG	60 (24.9)	75 (30.49)	1
AG	119 (49.38)	117 (47.56)	1.27 (0.83-1.94)
AA	62 (25.73)	54 (21.95)	1.44 (0.87-2.36)
AG or AA	181 (75.1)	171 (69.51)	1.32 (0.89-1.97)

Interaction between COMT and cyclin D1 in breast cancer

Table 2. Interaction between COMT-Met108/158Val and Cyclin D1-Pro241Pro SNPs. The genotypes were combined and categorized in four different groups for interaction analyses, and odds ratio and confidence intervals were calculated relative to the reference genotype [(COMTMet^L/Met^L) AND (CyclinD1^{GG})]. The analyses of the whole group and premenopausal subgroup are included.

COMT Genotype	CyclinD1 Genotype	Cases n (%)	Controls n (%)	OR (95% CI)
Whole data set				
Met^L/Met^L	GG	21 (5.28)	40 (10.75)	1
Met^L/Met^L	AA+AG	70 (17.59)	65 (17.47)	2.04 (1.09-3.81)
Val^H / Val^H + Met^L/Val^H	GG	82 (20.6)	74 (19.89)	2.07 (1.12-3.83)
Val^H / Val^H + Met^L/Val^H	AA+AG	225 (56.53)	193 (51.88)	2.21 (1.26-3.88)
Pre-menopausal				
Met^L/Met^L	GG	9 (3.73)	29 (11.79)	1
Met^L/Met^L	AA+AG	38 (15.77)	42 (17.07)	2.83 (1.19-6.77)
Val^H / Val^H + Met^L/Val^H	GG	51 (21.16)	46 (18.70)	3.345 (1.43-7.85)
Val^H / Val^H + Met^L/Val^H	AA+AG	143 (59.34)	129 (52.44)	3.49 (1.59-7.68)

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Legends

Figure 1. Graphical illustration of individual and interactive statistical analysis of COMT and Cyclin D1 SNPs both in whole sample and in premenopausal sub-group. For univariate analyses Met^L/Met^L and $CyclinD1^{GG}$ were used as reference genotypes for COMT and cyclin D1 SNPs, respectively. The genotypes were combined and categorized in four different groups for interaction analyses, and odds ratio and confidence intervals were calculated relative to the reference genotype [($COMTMet^L/Met^L$) AND ($CyclinD1^{GG}$)]. Rectangles represent the odds ratio, and error bars represent the 95% confidence interval. Black and grey rectangles represent the odds ratios for reference genotypes and other genotypes, respectively. Solid and dashed error bars indicate statistically significant and non-significant odds ratios, respectively.

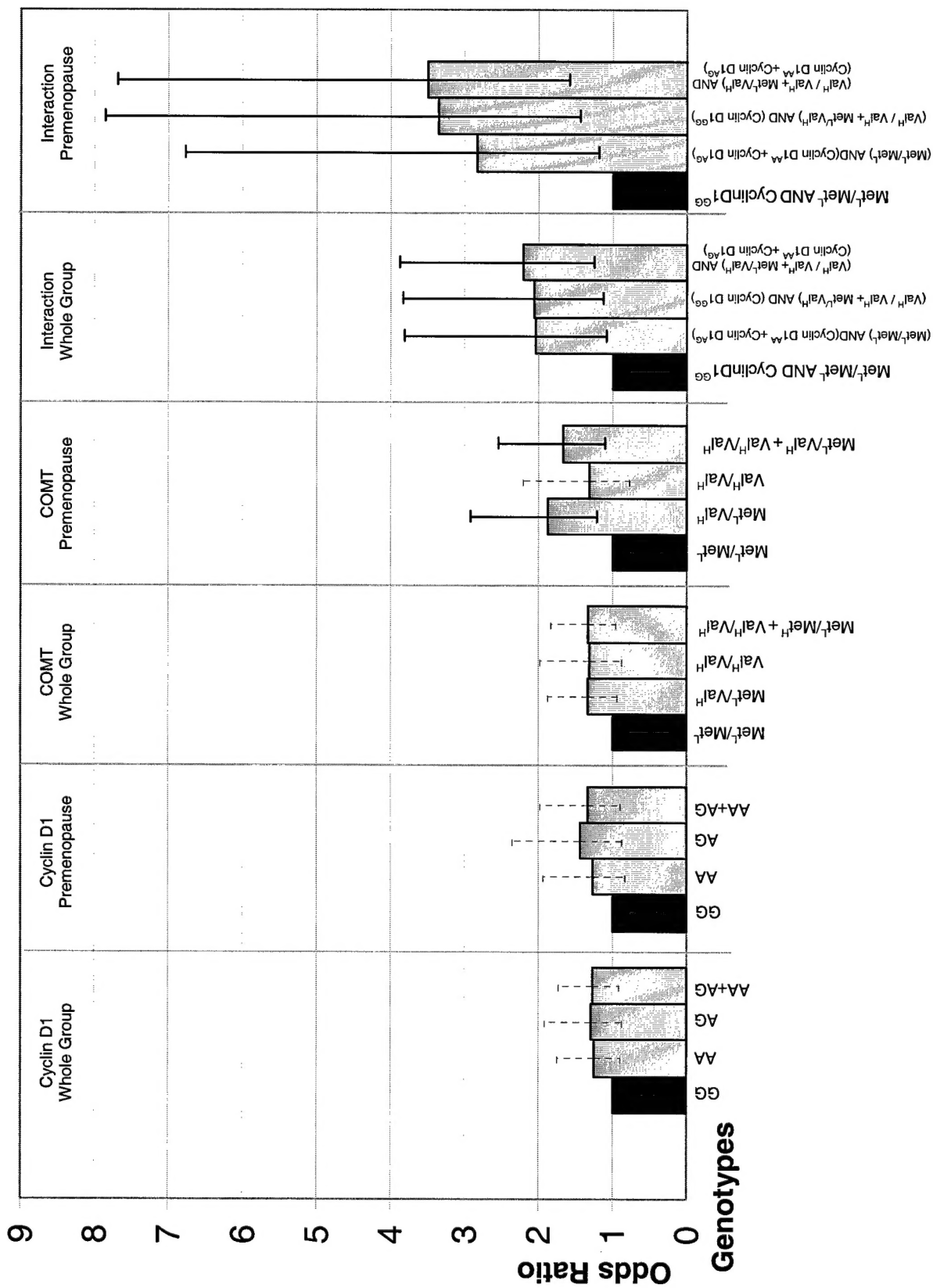**Figure 2.** Model for interaction between COMT and Cyclin D1 polymorphisms.

Figure 1.

Interaction between COMT and cyclin D1 in breast cancer

Figure 2.